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# RAPID ATTAINMENT OF A DOUBLED HAPLOID LINE FROM TRANSGENIC MAIZE (*ZEA MAYS* L.) PLANTS BY MEANS OF ANTHER CULTURE

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## Summary

We present a strategy for establishing a transgenic doubled haploid maize line from heterozygous transgenic material by means of anther culture. Compared to conventional inbreeding, the *in vitro* androgenesis technique enables a faster generation of virtually fully homozygous lines. Since the androgenic response is highly genotype-dependent, we crossed transgenic, non-androgenic plants carrying a herbicide resistance marker gene (*pat*, encoding for phosphinothricin acetyl transferase) with a highly androgenic genotype. The transgenic progenies were used as donor plants for anther culture. One transgenic and three non-transgenic doubled haploid lines have been established within approximately 1 yr. The homozygosity of all four doubled haploid lines was tested by analysis of simple sequence repeat (SSR) markers at 19 different loci. Polymorphisms were found between the lines but not within the lines, indicating the homozygous nature of the entire plant genome gained by anther culture. Southern blot analysis revealed that the transgenic donor plants and their doubled haploid progeny exhibited the same integration pattern of the *pat* gene. No segregation of the herbicide resistance trait has been observed among the progeny of the transgenic doubled haploid line.

*Key words:* androgenesis; homozygosity; SSR; *pat* gene; herbicide resistance.

## Introduction

*In vitro* androgenesis, the formation of fully homozygous plants from microspores, can dramatically speed up the development of inbred lines compared to conventional inbreeding. The initial protocols for the maize anther culture procedure (Kuo et al., 1978; Genovesi and Collins, 1982) continue to be improved. The main factors determining the efficiency of the establishment of doubled haploid (DH) lines are the androgenic response (Büter et al., 1991, 1993), the rate of chromosome doubling (Wan et al., 1991; Saisingtong et al., 1996b; Barnabás et al., 1999), and the efficiency of fertile plant regeneration. Murigneux et al. (1993a, b) demonstrated the homozygous nature of DH material generated by direct regeneration of gametic embryos, i.e., regeneration without an intervening callus phase, by analyzing phenotypic and molecular (RFLP) markers. Furthermore, the spectrum of genotypes showing androgenic responsiveness has been broadened and it was demonstrated that androgenic responsiveness can be transmitted to recalcitrant genotypes by inter-crossing (Jumpatong et al., 1996; Saisingtong et al., 1996a).

At the same time, protocols for maize transformation using various DNA delivery techniques and explants were developed (reviewed by Armstrong, 1999). Many protocols are based on the transformation of heterozygous material, which generally shows a higher transformation efficiency than inbred lines (Finer et al., 1992; Frame et al., 1994; Register et al., 1994; Brettschneider et al., 1997; Petolino et al., 2000). Consequently, the resulting progenies segregate for the transgenic trait but also for the genetic background, resulting in a high degree of variation, which may mask mild phenotypic effects of transgene expression. The establishment of transgenic and fully homozygous lines from such material requires seven generations (i.e., 7 yr) of self-pollination or back-crossing and selection for the transgenic trait in each generation.

In cases where the transformation of inbred lines is not feasible, the combination of genetic transformation and DH techniques could overcome this problem. Whereas attempts made to transfer foreign genes into microspores led to stable expression in barley (Yao et al., 1997; Carlson et al., 2001), oil seed rape (Fukoka et al., 1998), and wheat (Mentewab et al., 1999), all attempts to transform maize microspores have not yet resulted in transgenic DH maize lines (Fennel and Hauptmann, 1992; Jardinaud et al., 1995a, b).

An alternative approach originally suggested for wheat by Kunz et al. (2000) was taken to obtain transgenic DH lines in two subsequent steps from anther cultures initiated with previously transformed heterozygous material. We generated donor material for anther culture by crossing heterozygous transgenic material, lacking androgenic responsiveness, with a highly androgenic genotype, in order to establish a homozygous transgenic line from the DH regenerants. Using *in vitro* haploid techniques, this can be achieved within approximately 1 yr, while conventional inbreeding takes place over seven to eight generations.

Murigneux et al. (1993a) suggested that traces of heterozygosity found in DH lines may be due to on somaclonal variation rather than the process of androgenesis. In our laboratory, we adopted an indirect regeneration procedure based on secondary embryogenesis,

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in order to augment the output of regenerants. The prolonged callus phase and the additional *in vitro* culture period may lead to the accumulation of mutations and thus to additional genetic variation among DH plants. In order to confirm the homozygous nature of the DH lines obtained, PCR-based simple sequence repeat (SSR) markers showing a high level of allelic polymorphism (Smith et al., 1997; Pejic et al., 1998; Senior et al., 1998) were applied.

## Materials and Methods

*Donor material for anther culture.* Maize plants were grown in the greenhouse at 25 ± 3°C (day) and 18 ± 3°C (night) with a photoperiod of 16 h. The transgenic heterozygous genotypes 109.2 and 116.1 expressing the *pat* (phosphinothricin acetyl transferase) gene, which confers resistance to the herbicide Basta, were obtained from R. Brettschneider (University of Hamburg, Germany). They were generated by particle bombardment of zygotic embryos isolated from the line H99 (yellow kernels) after pollination with A188 (white kernels). The transgenic regenerants were back-crossed to A188. This material was segregated for the marker's kernel color and herbicide resistance. The highly androgenic genotype ETH-M82 (derived from a cross between ETH-M80 and ETH-M72) was pollinated with Basta-resistant individuals of 109.2 and 116.1, respectively. Anther donor plants were again selected by spraying their progenies with Basta.

As positive and negative controls for androgenic response, plants from the genotypes ETH-M82, 116.1 and 109.2, respectively, were chosen.

*DH line establishment by anther culture.* The protocol and media used for anther culture were as described by Saisingtong et al. (1996b): tassels of the selected donor plants containing microspores at a late uninucleated to early binucleated stage were harvested and subjected to a cold stress at 9°C for 10–14 d. Spikelets were then surface-sterilized for 10 min with a 2.5% (w/v) sodium hypochlorite solution supplemented with 0.1% (v/v) detergent (Mucasol, Merz & Co, Frankfurt, Germany). Isolated anthers were incubated in the dark at 14°C in liquid induction medium (IML; Saisingtong et al., 1996b) containing 250 mg $l^{-1}$ colchicine. After 1 wk, anthers were transferred to semisolid induction medium lacking colchicine (IMSS; Saisingtong et al., 1996b) and cultivated in the dark at 27°C for approximately 4 wk until the emergence of gametic embryos. For proliferation of the material, secondary embryogenesis was induced according to a protocol originally developed for zygotic embryos (Brettschneider et al., 1997). The gametic embryos were transferred to N6 basal medium supplemented with 20 g $l^{-1}$ sucrose, 3 g $l^{-1}$ phytagel, and 1 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), and cultivated in the dark at 27°C for 4 wk. For plant regeneration, embryogenic calluses were transferred to MS medium (Murashige and Skoog, 1962) supplemented with 3 mg $l^{-1}$ phytagel and 30 g $l^{-1}$ sucrose and exposed to light at 27°C; regenerated plantlets (G2 generation) were explanted to soil when their shoots were approximately 8 cm long. To establish a DH line (G3 generation, Fig. 1), regenerants were self-pollinated whenever possible. In parallel, ETH-M82 was pollinated by the G2 plants (G3′ generation, Fig. 1) to ensure the availability of material for segregation analysis of the transgenic trait.

*Selection of Basta-resistant plants.* Plants were sprayed at the three- to four-leaf stage with a 0.13% (v/v) solution of Basta (Bayer CropScience, Germany) supplemented with 0.5% (v/v) Tween 20. The final concentration of the active compound was 250 mg $l^{-1}$ bialaphos. The treatment was repeated after 3 d, and the herbicide resistance of the plants was assessed 3 d later. The same procedure was performed for transgenic 116.1 and non-transgenic plants as positive and negative controls, respectively.

*Southern hybridization.* Plant genomic DNA was extracted from lyophilized leaves according to the protocol of Hoisington et al. (1999) and 20 μg were digested with the enzymes *Eco*RI and *Hin*dIII. This enzyme combination cuts out the entire marker construct, including the 35S promoter and the *pat* gene. The resulting DNA fragments were separated on a 0.7% agarose gel, transferred to a nylon membrane (Schleicher & Schuell, Germany) by alkaline transfer (Sambrook et al., 1989), and hybridized with a digoxigenin-labeled PCR fragment of the *pat* gene. The hybridization was performed with the Roche™ DIG detection kit following the instructions of the supplier. For signal detection, the membrane was exposed to X-ray film (Kodak BioMax MR-1) for 45–90 min at ambient temperature.

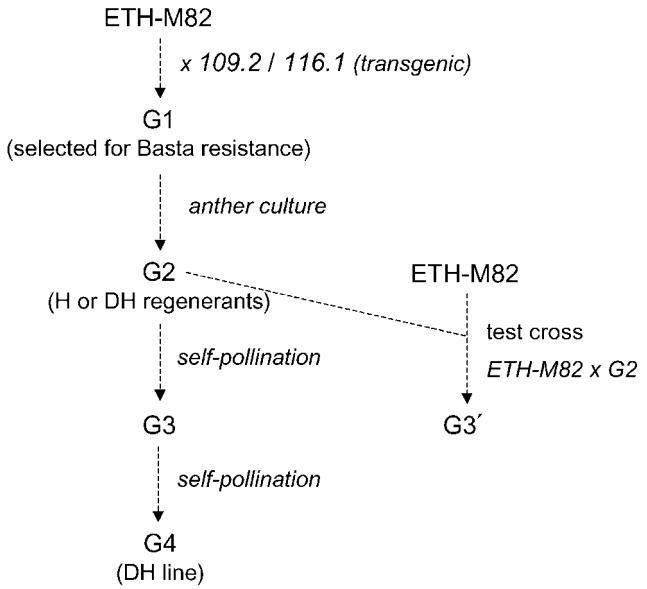


Fig. 1. Strategy for the establishment of a pure transgenic maize line by anther culture. Transgenic anther donor plants (G1) were selected from a cross between androgenetic responsive material (ETH-M82) and transgenic genotypes (109.2 and 116.1). Doubled haploid (DH) regenerants (G2) were self-pollinated to establish DH lines (G3, G4); a test-cross (G3′) allowed assessment of the homozygosity of G2.

The hybridization probe was synthesized and labeled with the Roche™ PCR DIG Probe Synthesis Kit using plasmid DNA as template and the following *pat*-specific oligonucleotide primers located at the positions 579–598 and 1092–1112 of the synthetic gene, respectively: *pat*1: 5′-GAG ACC AGT TGA GAT TAG GCC-3′; *pat*2: 5′-ATC TGG GTA ACT GGC CTA ACT-3′.

The PCR conditions were as follows: one denaturation step (94°C, 2 min) followed by 30 cycles of primer annealing (59°C, 30 s), elongation (72°C, 30 s) and denaturation (94°C, 30 s), and a final elongation step (72°C, 10 min).

*SSR analysis.* The doubled haploid lines (G4 generation) originated from anther culture were analyzed for homozygosity by the SSR method. The protocol for maize SSRs described by the CIMMYT Applied Molecular Genetics Laboratory (Hoisington et al., 1999) was adapted as follows: 100 ng of genomic template DNA per individual were used per PCR reaction. The PCR program consisted of one initial cycle of denaturation (94°C, 2 min), followed by 30 cycles of denaturation (94°C, 30 s), primer annealing (54°C, 1 min) and elongation (72°C, 2 min), followed by a final elongation (72°C, 5 min). The amplification product was run on a 4% (w/v) agarose gel containing 2% (w/v) Resophor agarose (Eurobio, France) and 2% (w/v) Agarose STG (Eurobio). A set of 96 SSR primers from the bnlg/phi/mmc/umc/dupssr series were tested for polymorphic band patterns on all four parentals (ETH-M80, ETH-M72, H99, and A188) from which donors for anther culture were originated. Primers that did not show at least one different band pattern between the four parentals were discarded. A final set of 19 primers (two for each chromosome except for chromosome 7) was chosen for further analysis: bnlg1112, bnlg1564, bnlg1338, bnlg2248, bnlg1447, bnlg1798, bnlg1217, bnlg1337, bnlg565, bnlg1695, bnlg249, bnlg1732, dupssr13, mmc0181, bnlg1863, phi028, bnlg244, bnlg1028, and umc1084. Of each doubled haploid line, 22 individuals were tested. To reduce the number of assays, the genomic DNA of two individuals was pooled.

## Results

*Androgenic response, plant regeneration efficiency, and establishment of DH lines.* From the crosses ETH-M82 × 109.2 and ETH-M82 × 116.1, 38 Basta-resistant plants were selected and used as donor plants for anther culture. A total of 6025 isolated

anthers yielded 837 gametic embryos which regenerated to 162 plantlets (G2 generation). The androgenic response of the crosses ETH-M82 × 116.1/109.2 resulted in an average yield of 17/25 embryos per 100 anthers with a very high variability within the repetitions (Table 1). From 100 of these embryos, an average of 19/27 plants were regenerated, resulting in a yield of 3/7 regenerants per 100 isolated anthers. Whereas the androgenic response was negative for the genotypes 109.2 and 116.1, the genotype ETH-M82 evidenced an extremely high yield of gametic embryos and an outstanding plant regeneration efficiency.

Only four DH descendants of 109.2, but none of 116.1, set seeds after self-pollination and gave rise to four DH lines (G3 generation). This overall low efficiency of DH line production was due to (1) the low survival rate of regenerants when transferred to soil, (2) developmental disorders such as the lack of either the male or female inflorescence, (3) male sterility, or (4) unsynchronized flowering.

While the parental genotypes 109.2 and 116.1 segregated for kernel color, the four DH lines had either white or yellow kernels exclusively.

*Inheritance of the* pat *gene*. From 82 herbicide-treated individuals of the G1 generation, 38 (46.3%) that exhibited resistance to Basta were selected as donors for anther culture. Southern analysis of 55 regenerants (G2 generation) revealed a positive hybridization signal for 14 (25.5%) plants.

The low number of seeds obtained by self-pollination of G2 individuals did not enable us to perform a segregation analysis in the G3 generation. Therefore, wild-type plants of the genotype ETH-M82 were pollinated with the transgenic and the three non-transgenic G2 individuals. This strategy allowed us to test 40 individuals of each cross (G3′ generation) for Basta resistance. All progenies from the non-transgenic G2 plants proved to be Basta-sensitive, while all progenies from the transgenic G2 plant were resistant. Sixteen of the resistant G3′ plants were also analyzed molecularly and gave a positive hybridization signal when probed for the *pat* gene.

Furthermore, the integration pattern of the foreign gene in all the tested transgenic individuals of the generations G2, G3, and G3′ was found to be identical to that of the transgenic parental line (109.2). A representative blot with two individuals of the G3 generation and the parental genotypes ETH-M82 and 109.2 is shown in Fig. 2.

The inheritance and expression of the transgenic trait in the established DH line was studied for a further generation. One transgenic G3 plant could be self-pollinated; of the resulting G4 generation, 43 individuals were sprayed with Basta and all of them proved to be resistant. As expected, the negative controls died off and the positive controls remained unaffected by the herbicide treatment.

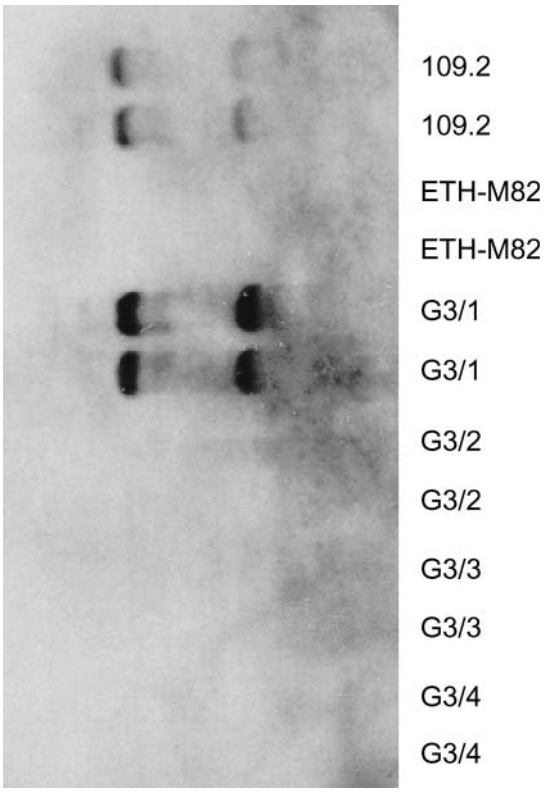


FIG. 2. Southern hybridization with a *pat* probe of *Hin*dIII–*Eco*RI-digested genomic DNA of G3 plants and the parental lines ETH-M82 and 109.2. Two individuals of the parental genotypes and two different G3 progenies of each of the four G2 plants have been tested; one of the four doubled haploid plants inherited the transgene.

*SSR analysis of DH lines*. A set of 19 polymorphic SSR markers was chosen to analyze the genetic variability between and within the four DH lines. From the 19 tested primer sets, nine revealed a different band pattern between at least two lines. Within the lines, no variation could be detected among the 22 individuals tested (Fig. 3).

TABLE 1

ANDROGENIC RESPONSE OF G1 AND CONTROLS: NUMBER OF EMBRYOS PER 100 ANTHERS, REGENERANTS PER 100 EMBRYOS, AND REGENERANTS PER 100 ANTHERS FOR THE GENOTYPES ETH-M82 × 109.2, ETH-M82 × 116.1, THE POSITIVE CONTROL GENOTYPE ETH-M82, AND THE NEGATIVE CONTROL GENOTYPES 109.2 AND 116.1 ARE SHOWN

| Genotype | Embryos per 100 anthers | Regenerants per 100 embryos | Regenerants per 100 anthers |
|---|---|---|---|
| ETH-M82 × 109.2 | 25.15 ± 45.94 | 27.46 ± 27.46 | 7.41 ± 15.80 |
| ETH-M82 × 116.1 | 16.56 ± 23.20 | 18.97 ± 30.27 | 2.73 ± 3.88 |
| ETH-M82 | 279.28 ± 263.49 | 50.22 ± 45.38 | 140.61 ± 98.33 |
| 109.2 | 0.00 ± 0.00 | – | – |
| 116.1 | 0.00 ± 0.00 | – | – |

Values are mean ± SD of *c.* 3000 anthers per genotype.

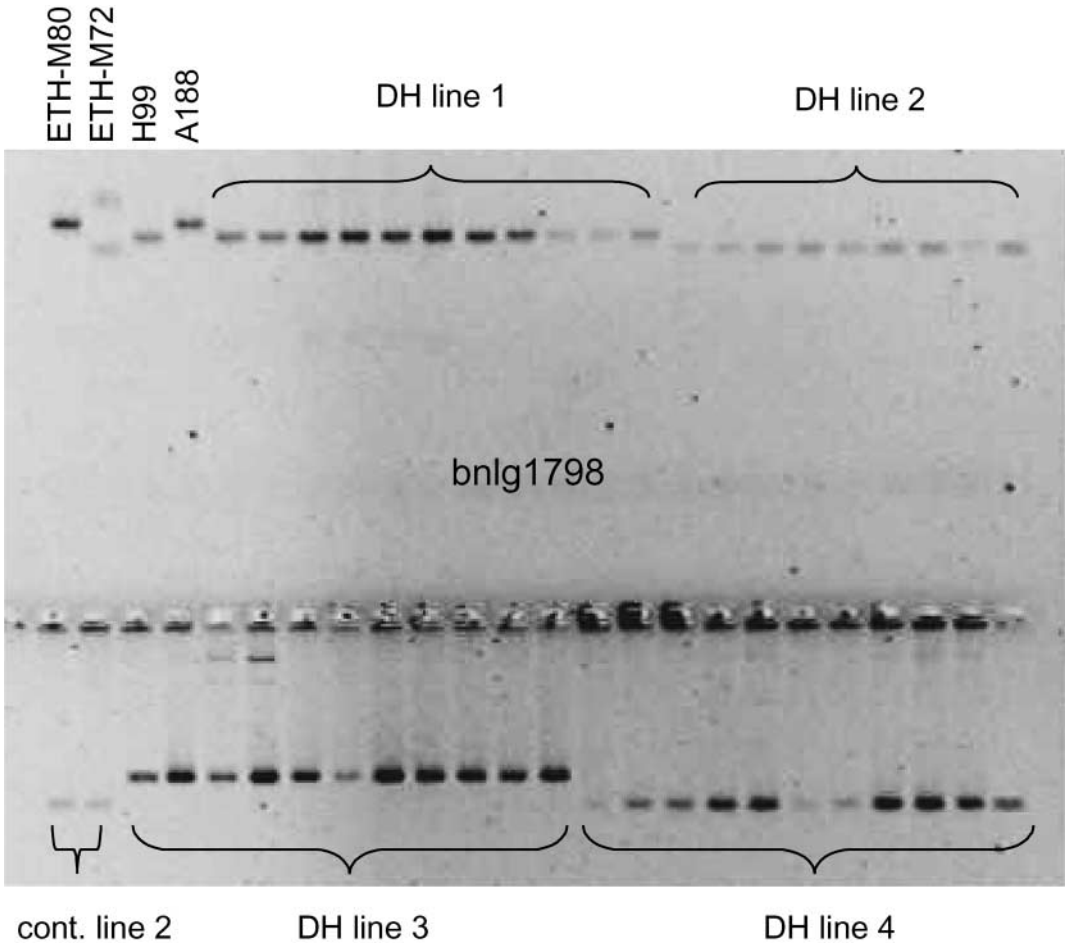


FIG. 3. SSR analysis of four DH lines (G4 generation) and their parentals. A representative analysis with the primer set bnlg1798 is shown. Of each DH line, 22 individuals (two pooled per assay) were investigated.

## DISCUSSION

We present a strategy for establishing fully homozygous transgenic maize lines from heterozygous transgenic donor material by means of anther culture. To demonstrate the inheritance and expression of a foreign gene in plants derived from the androgenic pathway, a herbicide resistance marker was used. The homozygous nature of the DH lines was confirmed by SSR marker analysis.

The androgenic response had been shown to be a polygenic heritable trait. Cowen et al. (1992), Murigneux et al. (1994), Beaumont et al. (1995), and Marhic et al. (1998) presented evidence of the existence of diverse chromosomal segments associated with responsiveness to *in vitro* androgenesis, regeneration efficiency, and the establishment of DH lines. While ETH-M82 is highly androgenic and the genotypes 116.1 and 109.2 do not show androgenic response, their crosses exhibit an intermediate responsiveness (Table 1). The high degree of variation in embryo yield and regeneration frequency within repetitions (Table 1) is due to the segregation of traits influencing the androgenic response in the G1 generation. Despite the considerable loss in efficiency compared to ETH-M82, the frequencies of embryo induction, plant regeneration, and establishment of DH lines were sufficient to obtain four independent lines. Further optimization of the plant regeneration procedure would certainly lead to a higher yield of fertile DH lines.

As detected by Southern hybridization, the parental genotype 109.2 contained at least two copies of the *pat* gene. The progenies in the G1 generation showed a 1:1 segregation rate of herbicide-resistant and -sensitive individuals, as was expected for a single heterozygous dominant trait, indicating a close linkage between these copies. The original hybridization pattern of 109.2 was preserved in all the transgenic individuals throughout the tested generations. The integration of the transgene was strictly associated with the Basta-resistant phenotype. On the other hand, non-transgenic plants which exhibited herbicide resistance ('escapes') have not been observed. This clearly demonstrates that the passage along the androgenic pathway did not cause any detectable rearrangements of the transgene at the DNA level, and the expression of the transgenic phenotype remained unaffected.

Since exclusively heterozygous herbicide-resistant plants were chosen as anther donors, we expected to find a 1:1 segregation between resistant and sensitive individuals of the G2 generation. In fact, only 25% of the 55 tested individuals inherited the *pat* gene, although G1 plants segregated in a Mendelian manner. This observation might indicate a selection pressure against transgenic cells during the anther culture procedure.

Because of the generally weak performance of *in vitro*-regenerated plants and the resulting low kernel yield of self-pollinated G2 plants, it was impossible to assess the uniformity of the G3 generation with regard to herbicide resistance. In order to prove the homozygosity of the G2 generation, we generated the G3′ generation by pollination of wild-type material by the G2 plants. The lack of any segregation in the G3′ generation with respect to herbicide resistance confirmed the occurrence of correct chromosome doubling.

Four lines with uniform kernel color were established by self-pollination of G3 individuals (G4 generation) but only one of them was herbicide-resistant. The purity of the transgenic DH line was further confirmed by spraying with Basta.

Additionally, all four DH lines were tested at 19 loci for uniformity by SSR markers. The homogeneous band pattern within the lines demonstrated the homozygous nature of the DHs. The passage through a 4-wk callus phase did not originate detectable DNA rearrangements due to somaclonal variations. Certainly, it was demonstrated by Müller et al. (1990) that a correlation exists between the duration of the callus culture phase and a high degree of DNA rearrangements. On the other hand, Brown et al. (1991) suggested that differentiation and organogenesis act as a selecting phase against novel DNA rearrangements, preventing high levels of variations in the regenerated plants.

Despite the presence of multiple transgene copies and the fully homozygous nature of the transgenic DH line, we found no indication for the occurrence of 'repeat-induced' (Matzke and Matzke, 1998; Jakowitsch et al., 1999) or 'homology-dependent' (Vaucheret et al., 1998; Fagard and Vaucheret, 2000) transgene silencing.

The application of DH techniques for establishing a homozygous transgenic line did not cause adverse effects on DNA structure or transgene expression and may thus be considered as an alternative to inbreeding via the sexual pathway.

Although the low yield of DH lines does not allow for a routine application of anther culture for breeding purposes, e.g., for the generation of large numbers of recombinant inbred lines, DH techniques may be advantageous in cases in which the rapid fixation of a transgene on a completely homozygous background (without the need to perform seven generations of self-pollination or back-crossing) is required. The expression of a transgene in a homogeneous background is a major requirement of basic researchers, who need to perform phenotypic analysis of the novel gene in an accurate way, without the 'noise' of any phenotypic variation given by an impure background. Androgenic material could be introgressed into the target material before the transformation is carried out. Alternatively, transgenic material could be crossed out to androgenic lines directly after regeneration.

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